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A Simple, Rapid ^{125}I Radioimmunoassay for the Detection of Barbiturates in Biological Fluids

A radioimmunoassay using a ^{3}H- or ^{125}I-labeled morphine antigen has been shown to be of practical value for the rapid detection of nanogram amounts of morphine and morphine analogs in biological fluids [*1-3*]. A radioimmunoassay for detection of barbiturates by conjugation of a secobarbital derivative to protein was also developed utilizing ^{14}C-labeled pentobarbital or barbital [*4,5*]. The present report describes our experiences with a practical radioimmunoassay for barbiturates employing a secobarbital derivative labeled with ^{125}I, as the antigen.

Materials

Barbiturate antigen—An iodinated derivative of secobarbital with a specific activity of 52 mCi/mg was employed in the assay.

Antibody to secobarbital—Secobarbital antibody was produced in goats according to the method of Spector and Flynn [*4*].

Ammonium sulfate—A saturated solution of $(NH_4)_2SO_4$ was prepared in deionized water without neutralization.

Phosphate-buffered saline (PBS, pH 7.2)—PBS was prepared as described by Catlin et al [*2*].

Normal goat serum—The serum was obtained from healthy adult male and female goats, sterile filtered, and used without further treatment.

Preparation of Barbiturate Standards

Normal human urine was collected from healthy adults, allowed to stand at 4°C overnight, decanted from any precipitates that formed, and the decanted urine was filtered through a sterile 0.45-μm Millipore® filter. Each urine sample that by radioimmunoassay (RIA) did not show an appreciable difference in counts per minute (CPM) from those obtained with buffer, was pooled and stored at 4°C for use.

Human blood was obtained by venipuncture into anticoagulant from healthy adult volunteers and the cells were separated by centrifugation. The individual plasmas were

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also tested for acceptability as described for urine. Acceptable plasmas were pooled, sterile filtered through a 0.45-μm Millipore® filter, and stored at −20°C until used.

Secobarbital (USP) prepared as a 20 μg/ml stock solution in H_2O, was stored at 4°C. The stock solution was then diluted in the pooled human urine or plasma to give the appropriate concentration of secobarbital per millilitre. Prepared standards were stored at 4°C. When employed, amobarbital, butabarbital, phenobarbital, pentobarbital, barbital, and hexobarbital standards were prepared in urine in the same manner.

Methods

The ^{125}I-labeled secobarbital derivative was diluted to 40,000 to 45,000 CPM/20 μl with PBS (efficiency of 43% for ^{125}I). A further 1:10 dilution in PBS was made and this material constituted the ^{125}I barbiturate antigen reagent. Goat antibarbiturate serum diluted in normal goat serum to a concentration sufficient to bind 82 to 85% of the above ^{125}I reagent was diluted with an equal volume of PBS for use as the antibody reagent. The assay was carried out as follows: 0.2 ml of the antibody reagent was added to each of a series of glass tubes (10 by 75 mm or 12 by 75 mm). After addition of 0.1 ml of sample to each tube, the contents were mixed on a Vortex® mixer. To the resulting mixture, 0.2 ml of the ^{125}I regeant was added followed by Vortex® mixing and incubation for 10 min to 5 h at ambient temperature. After the appropriate incubation period, 0.5 ml of saturated $(NH_4)_2SO_4$ solution was added to all tubes and precipitation was carried out for 10 min at ambient temperature. The tubes were then centrifuged in a swinging bucket rotor at 2500 × *g* for 10 min. 0.5 ml of each supernate was removed and transferred to standard counting vials and counted for 1 min in a Nuclear Chicago gamma scintillation counter Model 1185 (efficiency for ^{125}I, 43%). For quantitation of the assay, a standard curve was prepared by assaying in triplicate standards containing 0, 25, 50, 100, 200, and 400 ng secobarbital (or other barbiturate standards) per ml. The average CPM of each standard was then plotted on the *Y*-axis and the barbiturate concentration on the *X*-axis. The CPM of each unknown sample was converted to nanograms of secobarbital equivalents per ml by use of the standard curve.

If the CPM value of an unknown urine specimen was higher than the CPM value for 100 ng/ml secobarbital standard, then quantitation was accomplished by diluting the urine specimen 1:10 and 1:100 in pooled urine which had previously been shown to give a value similar to that of the negative urine standard. The diluted urine was assayed and the dilution which gave a value within the linear portion of the curve was used to calculate the secobarbital equivalents per ml of urine present.

Alternatively, quantitation could be accomplished by assaying 0.1 ml, 0.05 ml, and 0.02-ml amounts of the undiluted or diluted unknown urine. The standards were tested in the usual manner using 0.1 ml. The result obtained with the volumes of urine which fell within the linear range of the standard curve was then used for quantitation.

Quantitation of plasma was accomplished by testing 0.1-ml amounts of the specimens and if the value obtained was outside the linear portion of the standard curve, the plasma was diluted in appropriate negative plasma blanks and the dilution which gave a value within the linear portion of the curve was used to calculate the secobarbital equivalents per ml of plasma present.

Results

Figure 1 depicts a standard curve derived from the average of 18 to 20 determinations at each point on the standard curve after incubation for 1 h at ambient temperature.

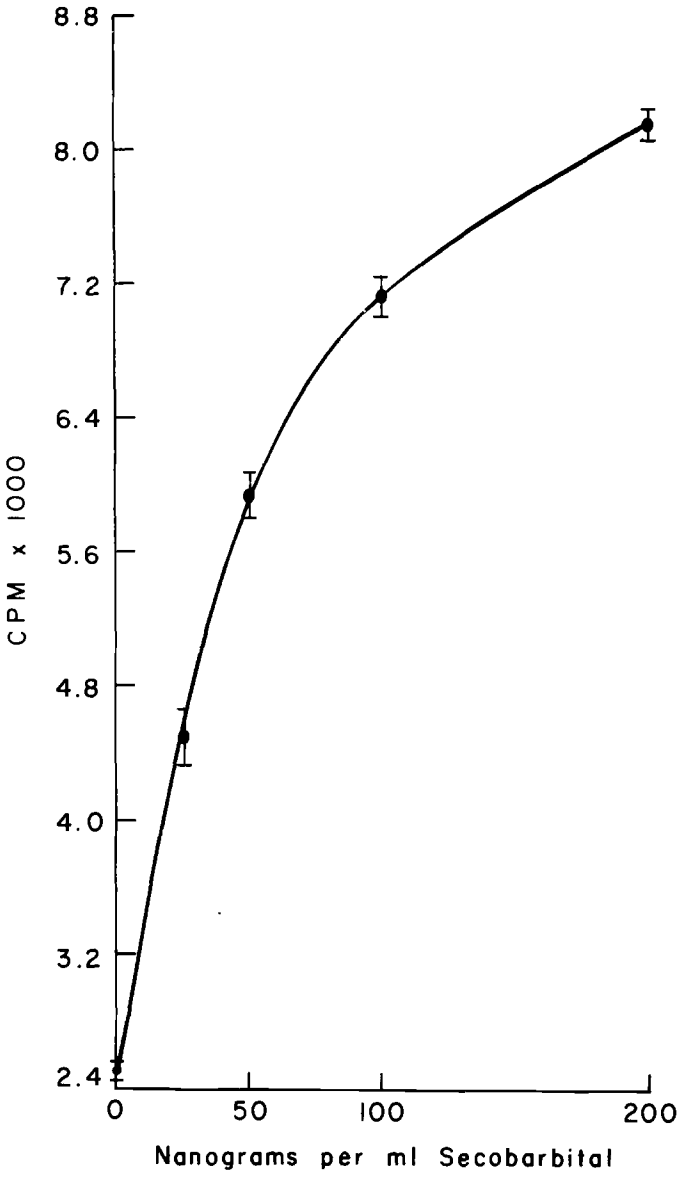

FIG. 1—*Reference curve obtained using secobarbital as the unlabeled standard.*

The standards were prepared in urine, but similar curves were obtained with standards prepared in plasma.

Shown in Fig. 2 are the results of tests to determine an acceptable minimal incubation time for the assay. After 10 min at ambient temperature, the curve is slightly but not significantly elevated over that seen at 1 h, which is not significantly higher than that at 5 h. All three response curves were similar. In fact, incubation for only 15 s, while

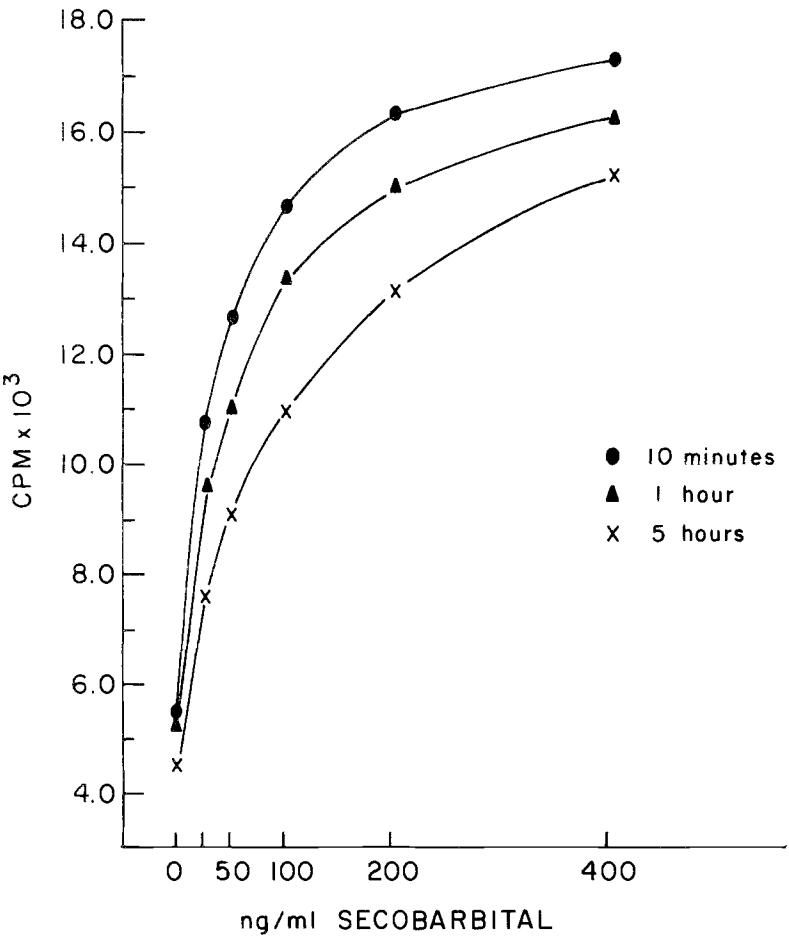

FIG. 2—*Response curve as a function of incubation time.*

showing an appreciably higher curve, results in an acceptable standard curve (not shown).

The remainder of the experimental work reported was carried out using a 1-h incubation period, since this was a convenient time interval for processing large numbers of samples.

The stability pattern of the ^{125}I reagent is directly related at 4°C or ambient temperature to radioactive decay, while at more elevated temperatures antigen degradation seems to play a part. This was illustrated when ^{125}I-labeled barbiturate antigen reagent and the corresponding matched antibody reagent were stored separately at 4, 24, 37, and 45°C. The test reagents were assayed immediately, and then at monthly intervals for up to 3 months, all reagents being brought to ambient temperature before assay. For each test, secobarbital standards prepared in urine were assayed in triplicate. In order to eliminate the variable of radioactivity decay, the results for each assay were plotted in the following manner: The average CPM obtained for each standard was divided into the

average CPM of the 0 standard and the quotient multiplied by 100 to give the percent of free ^{125}I secobarbital antigen at each secobarbital concentration, in relation to that free when no secobarbital was present.

The curves obtained at the time the reagents were set up for the stability experiment and after three months at the four temperatures (4, 24, 37, and 45°C) are shown in Fig. 3. The percent binding curves for the reagents held at 4 and 24°C were comparable to that

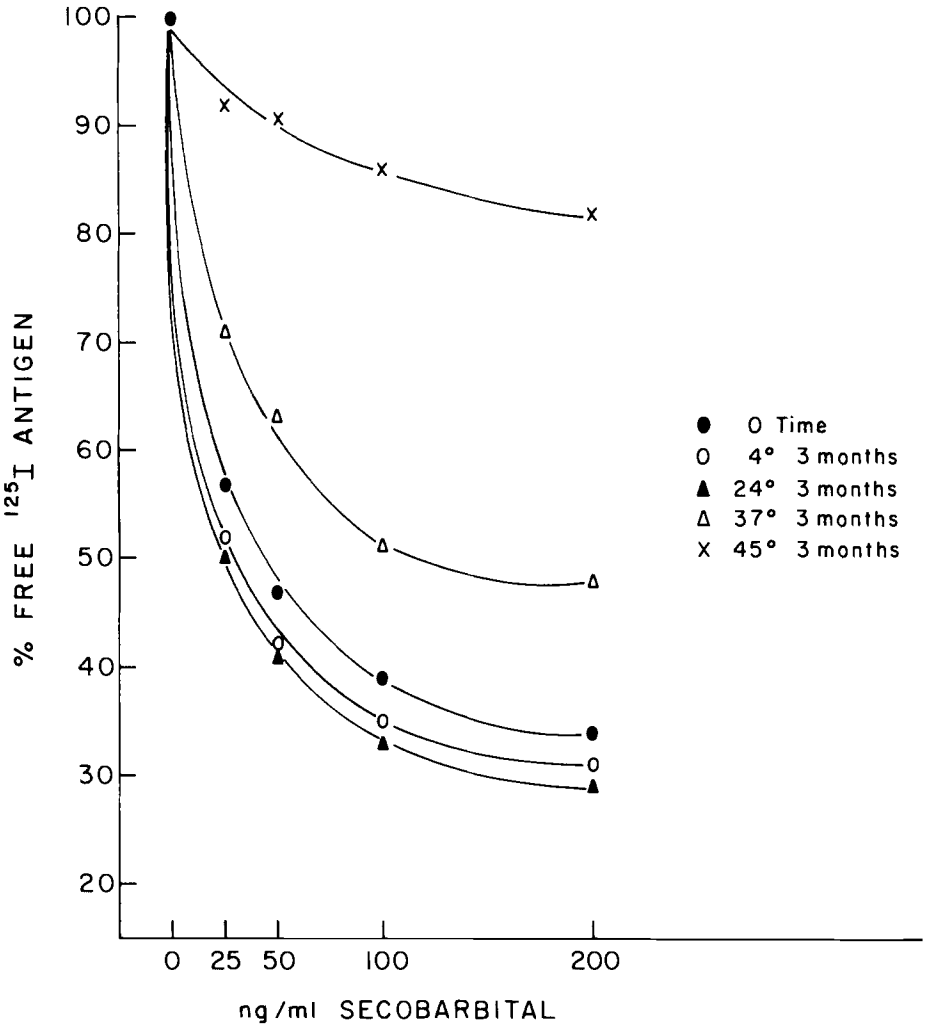

FIG. 3—*Effect of temperature and time on the stability of the ^{125}I barbiturate test reagents.*

obtained initially. The slope of the lines at these temperatures was almost identical to that when the reagents were first tested. This indicates that immunologically the reagents were substantially unchanged over the three-month period of storage at these temperatures. A slight flattening of the slope was observed at the three-month interval when the regeants

were held at 37°C, indicating that some degradation was occurring. This trend was accelerated by storage at 45°C. The reagents were still acceptable after three months at 37°C, but after three months at 45°C the standard curve was barely usable.

To determine which (or both) of the reagents incubated at temperatures above 24°C was becoming unstable, the reagents of the previous experiment after 92 days storage at the various temperatures were tested in the following manner: (1) barbiturate antibody held at 4°C was assayed using ^{125}I-labeled reagent held at 4, 37, and 45°C, and (2) barbiturate antibody held at 37 and 45°C was assayed using ^{125}I-labeled reagent held at 4 and at 45°C. The results shown in Fig. 4 are plotted with the *Y*-axis representing the

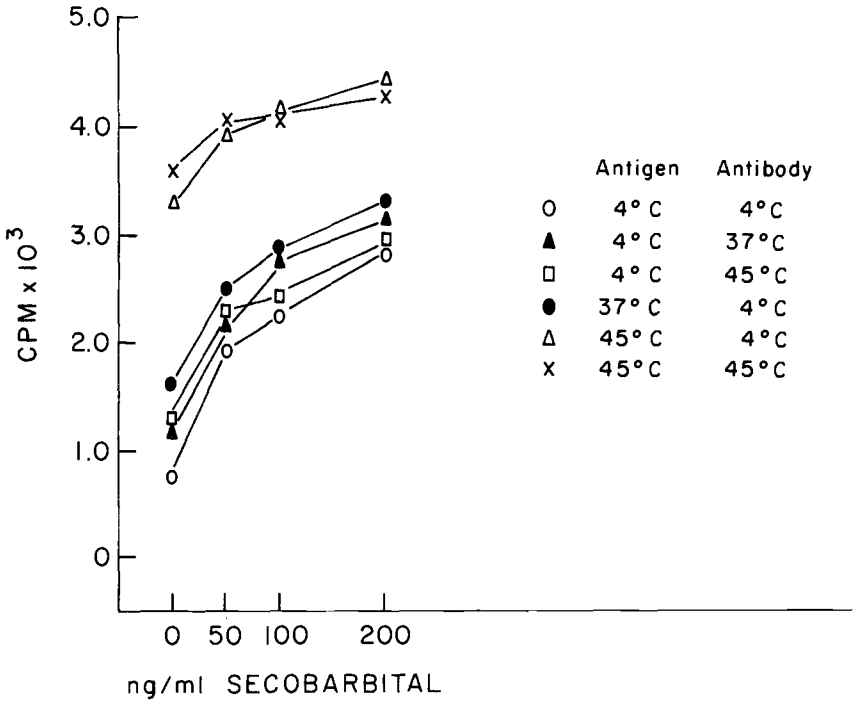

FIG. 4—*Effect of temperature on the stability of the ^{125}I secobarbital antigen.*

average CPM and the *X*-axis the concentration of unlabeled secobarbital.

When the ^{125}I antigen was incubated at 4°C and antibody at 4, 37, or 45°C, or when the ^{125}I antigen was incubated at 37°C and the antibody at 4°C, typical standard curves were obtained. However, incubation of the ^{125}I antigen at 45°C and antibody at 4 or 45°C resulted in virtually no displacement of labeled ^{125}I antigen from antibody by the unlabeled secobarbital. The higher initial counts under these conditions are probably due to the loss of antibody binding capacity by the ^{125}I antigen.

To evaluate the sensitivity level and the specificity of the RIA for barbiturates, urines or plasmas or both were obtained from the following populations:

(1) individuals who could have been using alcohol, nicotine, and/or caffeine ("normal" population);
(2) individuals known to be receiving specific drugs other than barbiturates; and
(3) individuals known to have received specific amounts of barbiturates.

It is essential in drug testing to establish a cutoff value which will yield as few false positives as possible. Therefore, to determine the range of values that one could expect in specimens obtained from individuals selected at random for a "normal" population, urines were collected from individuals in the first population and tested for the degree of reactivity in the assay. This population consisted of 214 individuals who presented themselves to the clinic of a large industrial concern for yearly routine or pre-employment physical examinations or because of minor illness. The samples were identified only by number and no attempt to obtain a medical history or follow-up was made.

As shown in Table 1, 198 specimens contained less than 50 ng secobarbital equivalents

TABLE 1—*"Apparent" concentration of secobarbital in urines of 214 individuals selected from a random, presumably normal population. Results are expressed in terms of secobarbital equivalents (SE) per ml of urine.*

Concentration of Secobarbital, ng SE/ml	No. of Individuals in the Range	% of Total
0-50	198	93
51-100	8	3
101-200	1	1
>200	7	3

(SE)/ml and eight contained less than 100 ng SE/ml. One specimen contained greater than 100 ng but less than 200 ng SE/ml, while seven specimens all contained greater than 200 ng SE/ml. Based on these results, a sensitivity of 100 ng secobarbital equivalents per ml urine was selected as the concentration to be used to distinguish between a "positive" and "negative" urine sample.

Values obtained with 23 plasma specimens from volunteers thought to be free from barbiturates for at least two weeks were less than 50 ng SE/ml. Thus, 50 ng SE/ml was selected as the cutoff point to distinguish between positive and negative plasma samples.

Since the RIA is designed as a diagnostic screening test for barbiturates, it is also critical to examine the effect of other drugs on the test system. Flynn and Spector [5] have shown that a number of nonbarbiturate substances similar in structure to the immunizing antigens do not cross-react in vitro. Because most drugs are excreted in the urine both unchanged and as metabolites, probably the most valid approach to detect potential cross-reactivity is to examine the urine of subjects receiving drugs orally, rather than to perform the tests on normal urine to which known amounts of drugs have been added.

Accordingly, the second group of volunteers on each of the drugs listed in Table 2 submitted one or more urine and plasma samples from 0 to 24 h after ingesting a clinically acceptable dose of each of the agents. The number of volunteers receiving each drug is small, but all urine or plasma contained less than the 50 ng SE/ml, so it is unlikely that any of these drugs significantly cross-react following therapeutic use.

The sensitivity of the radioimmunoassay for measurement of barbiturates in urine and plasma was ascertained by obtaining urine and plasma specimens from volunteers following oral administration of a clinically acceptable dose of butabarbital, phenobarbital, pentobarbital, secobarbital, aprobarbital, and barbital. These results are shown in Table 3.

TABLE 2—*Volunteers each received standard dose orally of the drugs listed below.*

Generic Name	Trade Name	Secobarbital Equivalents[a], ng/ml
Chlordiazepoxide	Librium®	<50
Diazepam	Valium®	<50
Chlorpromazine	Thorazine®	<50
Oxyphenbutazone	Tandearil®	<50
Phenylbutazone	Butazolidine®	<50
Aminopyrine	Pyramidon®	<50
Diphenylhydantoin	Dilantin®	<50
Trifluoperazine	Stelazine®	<50
Caffeine	...	<50
Glutethimide	Doriden® [b]	<50
Promethazine	Phenegan®	<50
Chloroquine	Aralen®	<50
Methaqualone	Quaalude®	<50
Methylprylon	Noludar®	<50

[a] Urine and plasma specimens collected 0, 1, and 4 after drug administration.
[b] Second volunteer after receiving glutethimide submitted urine and plasma collected 0, 1, 4, 8, 12 and 24 h after dose administration.

Evaluation of the urines from this controlled study with various barbiturates indicated that none of the urines from this third group of volunteers contained more than 26 ng secobarbital equivalents per ml prior to drug administration, while following medication, each of the barbiturates was readily detected in urine for at least 72 h. Similar results were obtained with plasma samples drawn 8, 12, 24, 48, and 72 h after drug administration.

All of the results thus far described were obtained using secobarbital as the unlabeled standard. Since both antibody and labeled antigen were prepared using a secobarbital derivative, it was of interest to determine what affinity other barbiturates would have for the antibody in relation to that of secobarbital. Additionally, it appeared important to determine what effect other barbiturates as unlabeled standards would have on the specificity and sensitivity of the assay when testing urines from "normal" individuals, as well as from volunteers receiving barbiturates.

Shown in Fig. 5 are a series of reference curves obtained using secobarbital, pentobarbital, butabarbital, amobarbital, phenobarbital, and barbital standards prepared in a pool of urine which had been shown previously to be negative when tested with secobarbital as the standard. From the data shown, it is evident that in order of reactivity secobarbital is the highest followed by pentobarbital, butabarbital, amobarbital, phenobarbital, and barbital. Not shown are results with hexobarbital, which did not displace any of the ^{125}I label even at concentrations of 1000 ng/ml.

In Table 4 are shown the relative reactivity of each barbiturate in nanograms per ml compared to 100 ng/ml secobarbital standard. One can see that barbital had 10%, phenobarbital 25%, amobarbital 35%, and butabarbital and pentobarbital approximately 45% of the relative activity of secobarbital. This would suggest that using, for example, phenobarbital as a standard in this assay, the sensitivity would increase by at least a factor of 4. However, the increase in sensitivity might result in a corresponding increase in false positive results. One hundred urines from the first population were, therefore, tested using all of the above barbiturate standards prepared by making dilutions in single urine pool. The ng/ml equivalents for each urine against each standard were then determined. The results of these tests are shown in Table 5.

TABLE 3—*Urine barbiturate levels as a function of time after receiving known dosages of selected barbiturates.*

Time, h	Barbiturate Concentration, ng SE/ml [a]								
	Phenobarbital, [b] 30 mg	Pentobarbital, 100 mg		Secobarbital, 100 mg		Barbital, 80 mg	Butabarbital, 100 mg		Aprobarbital, 80 mg
		1	2	1	2		1	2	
0	7	1	6	4	7	18	26	0	4
4-8	240	...	>800	>800	>800	>800	340	290	720
12-24	250	570	>800	>800	>800	>800	350	260	>800
24-48	220	260	>800	670	>800	>800	470	330	>800
48-72	460	480	>800	>800	>800	600	310	240	680

[a] Nanograms of secobarbital equivalents per ml of urine.
[b] Single dose administered orally.

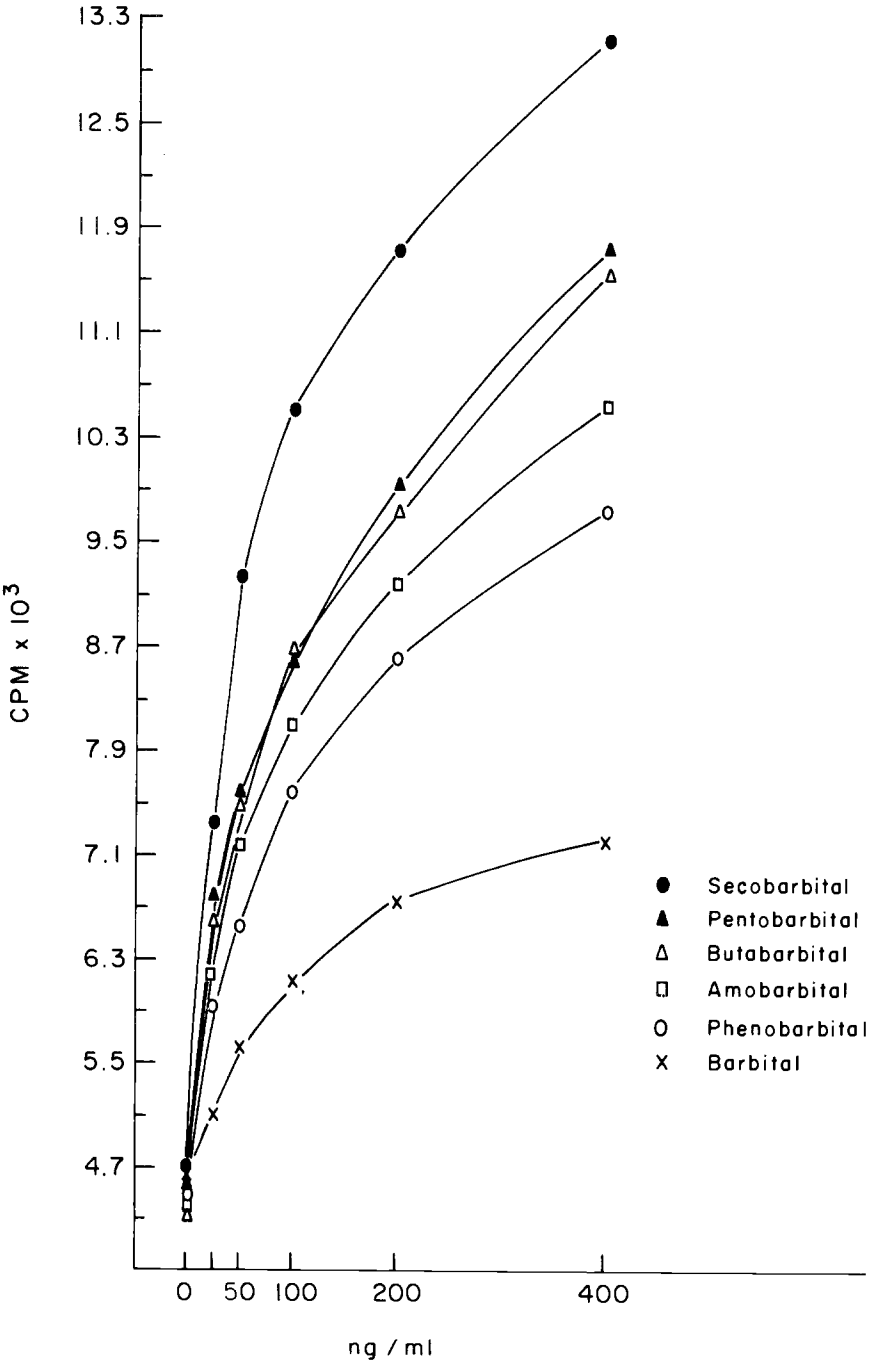

FIG. 5—*Response curve as a function of the unlabeled barbiturate standard.*

TABLE 4—*Relative activity of five barbiturates when compared with 100 ng/ml secobarbital standard.*

Secobarbital, ng SE/ml [a]	100
Pentobarbital, ng SE/ml	45
Butabarbital, ng SE/ml	45
Amobarbital, ng SE/ml	35
Phenobarbital, ng SE/ml	25
Barbital, ng SE/ml	10

[a] Nanograms of secobarbital equivalents per 1 ml of urine.

TABLE 5—*Percentage of urines from "normal" random population giving positive results by RIA when tested using six barbiturate standards.*

	Range, ng/ml		
Barbiturate	0-100	101-500	>500
Secobarbital	97	2	1
Butabarbital	94	3	3
Pentobarbital	96	1	3
Amobarbital	93	4	3
Phenobarbital	93	3	4
Barbital	90	3	7

The proportion of negative urines found using secobarbital (<100 ng equivalents/ml) as the standard was 97%, butabarbital 94%, pentobarbital 96%, amobarbital 93%, phenobarbital 93%, and barbital 90%. The urines positive above 100 ng/ml level using secobarbital as the standard were all above the 500 ng/ml level using the other standards. However, urines giving positive values with the other barbiturates were obviously in some cases negative when the secobarbital standard was employed.

In order to show the increased sensitivity of the assay in detecting barbiturates by using standards with less affinity than secobarbital for the antibody-labeled antigen complex, one subject was given 80 mg of aprobarbital orally. Urines were collected before drug administration and every 24 h starting 48 h after drug administration for up to ten days.

Each urine was tested undiluted and at a 1:10 and at a 1:100 dilution for quantitation using the six barbiturate standards. The dilutions were made using a pool of normal urine shown previously to give 0 ng/ml reading when assayed against the six barbiturates. Fresh standards were also prepared using the same urine pool for preparing dilutions. The ng/ml equivalent of each urine collected at the different time intervals was then determined for each standard and the results are presented in Table 6.

It was possible using a 100 ng/ml cutoff to detect barbiturates for the ten-day period. As the relative reactivity for each standard in relation to secobarbital was lowered, the absolute ng/ml value at each time period almost invariably increased.

TABLE 6—*Relative sensitivity of six barbiturate standards for detection of barbiturates in urine specimens.* [a]

Time, h	Barbiturate, ng/ml					
	Seco-barbital	Pento-barbital	Buta-barbital	Amo-barbital	Pheno-barbital	Barbital
0	5 [b]	15	15	20	20	50
48	800	2350	2650	3800	5650	23,500
72	550	1500	1750	2500	3650	15,000
96	600	2650	1950	2100	3750	16,000
120	350	1200	1350	1850	2650	15,000
144	300	900	900	1350	1850	11,500
168	350	900	950	1350	2050	10,000
192	250	700	700	1050	1450	10,000
216	200	550	550	600	1100	6,000
240	200	450	450	600	800	4,400

[a] Volunteer received a single 80-mg dose of aprobarbital orally and submitted urine specimens at the times specified following drug administration.

[b] Nanogram equivalents per ml based on use of the standard curve for each barbiturate listed.

Discussion

The data presented clearly show that the radioimmunoassay for barbiturates described is a sensitive assay which appears to be specific for detecting barbiturates. The sensitivity of this assay is advantageous in at least two ways: (1) a negative result clearly indicates that barbiturates are not present and that additional testing is not necessary, and (2) detection of barbiturates for long periods of time is possible.

Additionally, the sensitivity of the radioimmunoassay can be adjusted by simply selecting the proper barbiturate as the standard.

Although we have not yet encountered drugs which may cause cross-reactions, there is always the possibility that additional experience with the test may reveal cross-reacting compounds. Therefore, to insure absolute identification of barbiturate usage and to identify which of the barbiturates is being used, nonserologic confirmatory tests should be employed.

Finally, from the data presented, it is apparent that this radioimmunoassay is not only sensitive but quite simple to carry out, can be completed in less than 1 h, and thus would seem quite suitable for large-scale testing of urine or serum specimens.

Summary

A simple, rapid radioimmunoassay employing ^{125}I-labeled secobarbital derivative has been developed and has been shown to be capable of detecting at the nanogram level a variety of barbiturates in urine as well as in plasma.

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